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Original Article

Morphological evaluation of apoptosis induced by salicylates in HT-1080 human fibrosarcoma cells

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ABSTRACT

Salicylates are a group of phenolic compounds that are found naturally in plants. They exert anti-inflammatory and pro-apoptosis activities. Three salicylates (acetyl salicylic acid, ASA; acetyl salicylate calcium, ASCa and salicylate calcium, SACa) were tested in human fibrosarcoma cells for their apoptotic activities in HT-1080 cells using both biochemical and morphological approaches. Salicylates-treated HT-1080 cells exhibited typical apoptotic features, including membrane blebbing, shrinkage of the cell and fragmentation into apoptotic bodies. The effects of the DNA labelling agent, BrdU, resulted in more rounder smaller and a smooth membrane in HT-1080 cells. These salicylate compounds demonstrated anti-proliferation and reduced the metabolic activities of HT-1080 cells by time- and concentration-dependent manners.

1. Introduction

Acetylsalicylic acid (ASA), or aspirin, and its precursor salicylic acid (SA) are common drugs which are associated with the β-D-salicin compound and are found abundantly in the medicinal willow tree [1]. The medicinal virtue of the willow has been well known since the Assyrians and Sumerians more than 6000 years ago [1]. Chemically, ASA and SA are classified as simple phenolic acids. These acids encompass different analogues that have a hydroxyl group at different positions on benzene ring moiety. Salicylates belong to non-steroidal anti-inflammatory drugs (NSAIDs) and exhibit multiple therapeutic activities such as anti-pyretic and anti-inflammatory action *via* the inhibition of COX-2 and its expression *via* NF-κB [2]. The activation of this transcription factor provides the potential link between inflammation and cancer. NF-κB formation is the consequence of an inflammatory microenvironment during malignant development. It up-regulates the expression of cancer-promoting cytokines, including IL-6 or TNF-α, and survival genes, such as Bcl-X_L [3]. Epidemiological-related studies have indicated that salicylates play a significant role as protective agents against types of breast, colorectal, gastric and oral cancers [4–7]. In addition, researchers *in vitro* indicated that hydroxybenzoate metal ion (Na^+, Li^+, Ca^{2+} and Zn^{2+}) complexes modulated the microenvironment of various cancer cell lines by inducing apoptosis [8–10]. The association of apoptosis is a mechanism contributory to how salicylates may prevent or act as anticancer agent [11–14].

Apoptosis is tightly regulated physiological mechanism that involves modulation of molecular biology, including caspases and kinases [15]. The regulation of apoptosis

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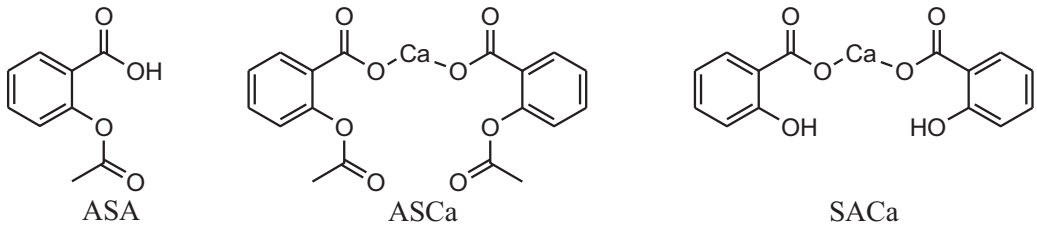


Fig. 1. Chemical structures of ASA, ASCa and SACa.

is critical to the pathogenesis of a number of diseases, including cancer [16]. Under normal conditions, both cell proliferation and apoptosis are balanced. However, if this balance is shifted in favour of an enhanced survival of damaged cells promote the carcinogenic process by allowing the survival of potentially neoplastic cells [17]. The significance of apoptosis has attracted research to explore the molecular signalling pathway mechanisms, including the initiation, mediation, execution and regulation of apoptosis. Intensive efforts have identified two major pathways for apoptosis. These are the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway) [18].

Morphologically, apoptosis was first characterised by changes in chromatin condensation, cellular shrinkage, budding and apoptotic body formation [19,20]. Scanning electron microscope is an important technique for detecting subcellular changes in apoptotic cells. These changes reflect cellular biochemical changes induced by extrinsic or intrinsic factors [18]. The morphological expression of drug-treated cell is a useful approach which enables an understanding of the changes caused by drugs. The aim of this research was to study the pro-apoptotic activities of 3 salicylates (Fig. 1) morphologically in HT-1080 fibrosarcoma cells and in the presence of 5-bromo,2-deoxy uridine (BrdU).

2. Materials and methods

ASA was obtained from Sigma, UK. ASCa and SACa compounds were prepared by acid base reaction of the corresponding starting materials ASA and SA (Sigma, UK), respectively. ASCa and SACa compounds were purified by crystallisation from water ethanol 40:60 mixture. 5-bromo,2-deoxy uridine (BrdU) was obtained from Sigma, UK. Stock solutions of chemical compounds were prepared at different concentrations in Dulbecco's Modified Eagle's Medium (DMEM).

The human fibrosarcoma cell line HT-1080 was cultured at different cell densities in DMEM medium contained 10% foetal calf-serum (FCS, Gibco, U.K), L-glutamine (Gibco, UK), 4% gantamicin solution (Gibco, UK), 1% L-glutamine (Gibco, UK), 0.1% hepes buffer (Gibco, UK), 0.1% sodium pyruvate (Gibco, UK), 0.1% ascorbic acid powder (Sigma–Aldrich, UK) and 1 ml/100 ml gentamycin solution. Cells were incubated at 37 °C in a humidified atmosphere and 5% CO_2. First the growth curve was performed to identify the exponential phase for drug treatment.

2.1. Cell viability assay

HT-1080 cells were seeded into 96-well plates at a density of 15×10^3 cells in 100 μl of media per well. Cells were left for 24 h to attach to the surface. Media was replaced with 100 μl containing different concentrations of salicylate compounds (0, 0.4, 0.8, 2, 4, 6, and 8 mM). Cells were allowed to grow in the presence of individual salicylate compound for 24, 48 and 72 h. In addition, BrdU (0, 200, 400, 600, and 1000 μM) was separately seeded in HT-1080 cell culture and cells were allowed to grow for 48 h. At the end of cell incubation, the media were replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, UK) reagent in free FCS DMEM and incubated for 60 min at 37 °C. Cells were then further incubated at the same conditions with MTTlysis buffer for 80 min before reading the absorption of formazane at 575 nm.

2.2. Cell proliferation assay

HT-1080 cells were seeded, cultured and allowed to settle as in the cell viability assay. Cultured cells were incubated with different concentrations of salicylates (0, 4 and 6 mM), containing 200 μM BrdU-labelling reagent and allowed cell to grow for 48 h. Cells were then fixed with FixDenat solution and incubated with anti-BrdU-horseradish peroxidise (HRP)-Fab antibody-fragments to bind the BrdU in the newly synthesised cellular DNA. The immune complexes were detected by using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and quantified by measuring the absorbance at 370 nm.

2.3. Cell cycle analysis

HT-1080 cells were seeded in T-25 flasks (Coster) at an initial density of 1×10^3 cells per flask. Cells were cultured as described in previous discussions before being treated with ASA, ASCa and SACa for 24 h. In addition, control experiments were performed in which no salicylate compounds were incorporated in the cultures. At the end of each treatment, the cells were harvested by trypsinization, were centrifuged and re-suspended in 200 μl of PBS. Aliquots (2 ml) of ice-cold 70% ethanol were then added and the cells were vortexed prior to being cooled to −20° for 30 min. Subsequently, 100 μl of RNase (Sigma, UK) at a concentration of 1 mg/ml and 100 μl of propidium iodide (Biovision Incorporate, CA, USA) at a concentration of 0.4 mg/ml was added prior to incubation at 37 °C for

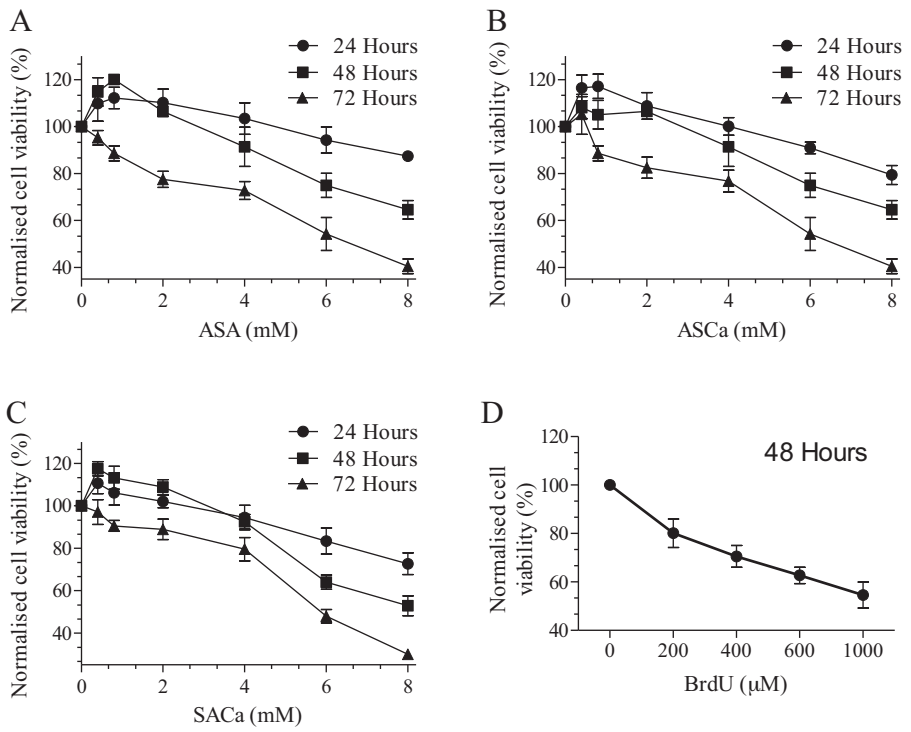


Fig. 2. Time- and concentration-response curves of treated HT-1080 cells with salicylate compounds. The data was derived from MTT cell viability assay, in 3 replicates, after HT-1080 cells cultured with 0–8 mM salicylate compounds for 24 h, 48 h, and 72 h under optimal culture conditions. (a) Cell viability after treatment with ASA, (b) cell viability after treatment with ASCa, (c) cell viability after treatment with SACa, (d) cell viability after treatment with BrdU for 48 h. The values (3 replicates) for farmazone absorption were calculated by normalising relative absorption values to that of the control. Thereby cell viability for control is 100% while treatment is either below or above 100%. Normalised values were calculated as ratios of ($Absorption_{control} - Absorption_{treatment}/Absorption_{control}$).

30 min. The cells were analysed using a FACS Calibur flow cytometer (Becton Dickinson, UK).

2.4. Scanning electron microscopy

HT-1080 cells were seeded in a 12-well plate containing microscopic slide cover slips at density of 15×10^3 cells, incubated for 48 h with the designated drug treatment before fixation where samples were treated for 1 h with the fixative (0.8% glutaraldehyde, 0.6% osmium tetroxide, 2 mM $CaCl_2$, and 0.2 M sucrose in 0.1 M sodium cacodylate buffer pH 7.4), washed for several times in PBS buffer and dehydrated with different concentrations of alcohol (30, 50, 70, 90, each for 5 min and 100% for 10 min twice). The dehydrated samples were then dried to the critical point in a Blazers CPD 030 using CO_2. Cell samples were mounted onto 12 mm 'Philips type' aluminium stubs using silver paint and then gold sputter coated in an Edwards S150B sputter coater. Finally, the samples were imaged using a Philips XL20 SEM under various magnifications ranging from 100× up to 2000×.

2.5. Statistical analysis

Data obtained in these experiments represent an average of 3 replicates which were evaluated using equal variance and paired Student's *t*-test (two-tails). Along with other statistical analyses, Graphpad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA) was mainly used.

3. Results

3.1. Cytotoxic effects of salicylates on the viability of HT-1080 cells

The cytotoxicity of ASA, ASCa and SACa compounds were tested by in *in vitro* culture growth media using HT-1080 human fibrosarcoma cells. HT-1080 cells were allowed to grow for 24, 48 and 72 h under optimal culture conditions that contain different concentrations of salicylate compounds. The results of MTT assays showed that the metabolic activities of the three salicylates exhibited biphasic effects; *i.e.* mitogenic and cytotoxic comparing to the untreated HT-1080 cells. At concentrations below approximately 2 mM and cultured for 24 and 48 h, HT-1080 cells showed an increase in the metabolic activities (Fig. 2A–C). However, the metabolic activity of salicylate-treated HT-1080 cells was decreased by treatment duration (72 h) and higher concentrations (2 mM or more) (Fig. 2A–C).

In addition, the effects of these three salicylate compounds on the metabolic activities of HT-1080 cells indicated that SACa caused more reduction in cell viability by 7–12% compared to both ASA and ASCa at 6 mM and 8 mM (Fig. 2A–C). Similar results were obtained at

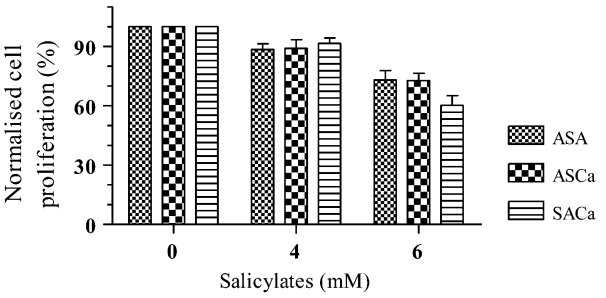


Fig. 3. The effect of 4 and 6 mM salicylates on HT-1080 cell proliferation. Cells were cultured under standard growth conditions for 48 h in 3 replicates. BrdU (200 μM) was incorporated in cell culture at the same time when individual salicylate was added.

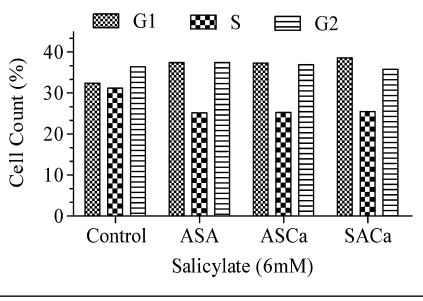
lower concentrations and in a similar pattern, whereas, these salicylates affected the viability of HT-1080 cells in time- and concentration manners. However, none of the treatments, particularly when cells were incubated for 48 and 72 h with 4, 6 and 8 mM showed significant differences ($p = 0.05$) between each pair. Fig. 2D shows the effect of 5-bromodeoxyuriding (BrdU) on the metabolic activities of HT-1080 cells. Fig. 2D shows a negative correlation between HT-1080 cells metabolic activity and BrdU concentrations. Therefore, 200 μM of BrdU was used to assess the cytotoxic effects of ASA, ASCa and SACa compounds on the proliferation of HT-1080 cells when treated for 48 h. Fig. 3 shows that ASA, ASCa and SACa compounds did not significantly ($p = 0.05$) differ in the reduction of the proliferation of HT-1080 cells when treated for 48 h.

3.2. The effect of salicylates on cell cycle

Fig. 4 shows that ASA, ASCa and SACa compounds induced similar modulation to the HT-1080 cell cycle. Incubation of HT-1080 cells with 6 mM salicylate modulated the cell cycle in a similar pattern. Thereby the number of cells increased by an average of approximately 17.5% at G0/G1 phase. In contrast, the number of cells at S-phase decreased by an average of approximately 18.3%. These effects are more apparent after treating HT-1080 cells for 24 h to individual salicylate and proceeded the characteristic development of a sub G0/G1 phase associated with the reduction of cell metabolic activity and proliferation, leading to apoptotic cell death.

3.3. Morphological characteristics of salicylate-treated HT-1080 cells

The morphological effects of the three salicylates; ASA, ASCa and SACa on HT-1080 cells were investigated using mainly scanning electron microscopy. HT-1080 was treated with different salicylates concentrations (0.4, 2, 6 and 8 mM) for 48 h under standard culture conditions. As 200 μM BrdU was used in determining cell proliferation, it was first necessary to investigate the effect of BrdU on the morphological properties of HT-1080. Fig. 5 compares the effect of 200 μM BrdU on HT-1080 cells before testing the effects of salicylates on cell's morphology. HT-1080 cells cultured with BrdU showing a clear difference in morphology compared to the control cells (Fig. 5B). HT-1080 cells cultured without BrdU displayed a typical flat cells with



Treatment	Cell Cycle phases (100%)		
	G1	S	G2
Control	32.4	31.2	36.4
ASA (6mM)	37.4	25.2	37.4
ASCa (6mM)	37.3	25.3	36.9
SACa (6mM)	38.6	25.5	35.8

Fig. 4. The distribution of cell cycle phases of salicylate-treated HT-1080 cells for 24 h.

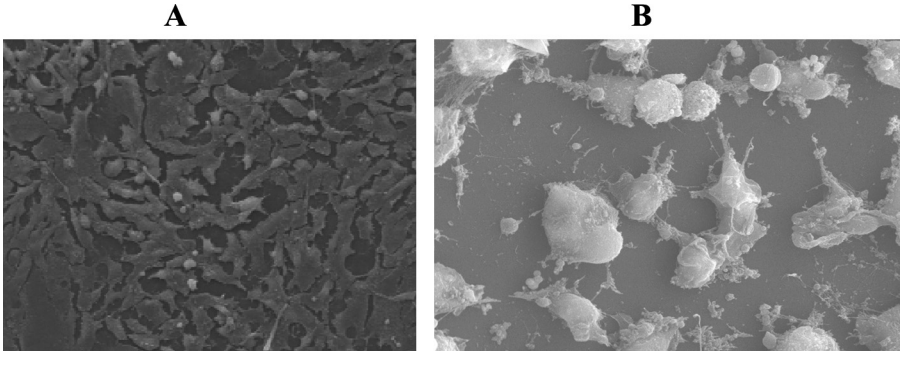


Fig. 5. The effect of 200 μM Brdu on the morphology of HT-1080 cells (A) without BrdU and (B) with BrdU. Magnification = 500×.

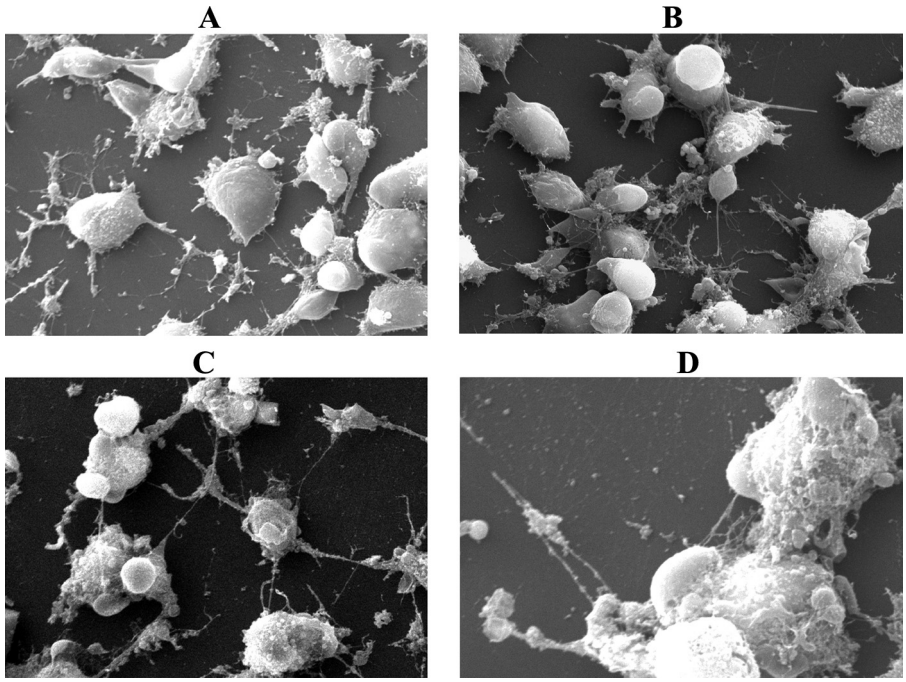


Fig. 6. The combined effect of salicylates and 200 μM BrdU on the morphology of HT-1080 cells cultured for 48 h (A) 0.4 mM ASA, (B) 0.4 mM ASCa, (C and D) 0.4 mM SACa (magnifications A–C = 500×; for D = 2000×).

the distinguishable feature of irregular shapes of neoplastic cells (Fig. 5A). It is interesting that the incorporation of BrdU has caused cells to curl up, with a round shape and appear to grow in small groups after 48 h of culturing (Fig. 5B). Cell cultures with BrdU were distinct of microvilliate and had anchors which assisted their pseudopodial attachments to indicate the viability of these cells.

Incorporation of salicylate compounds in cultured HT-1080 cells and in the presence of 200 μM BrdU also caused cells to curl up. Fig. 6 indicates that at 0.4 mM concentration, HT-1080 cell populations retained their viabilities, as indicated with the appearance of anchors, pseudopodial attachments, and rough or hairy of the cell surface membrane. Approximately 15% of cells treated with 0.4 mM ASA were rounded, curled up and lost their anchors suggesting that they underwent apoptotic cell death (Fig. 6A). Apoptosis is also apparent from the presence of apoptotic bodies. Some of these cells are rounded with a smooth cell surface but others were hairy which may reflect the stage of apoptosis. At the same concentration, both the ASCa (Fig. 6B) and SACa (Fig. 6C) compounds gave different morphological features compared to untreated HT-1080 cells (Fig. 5A) and salicylic acid treatment (Fig. 6A).

The main features that distinguish HT-1080 treated with salicylate compounds are cell sizes and the characteristics of their shapes. ASCa caused shrinkage of cell size (Fig. 6B) compared to control containing of 200 μM BrdU (Fig. 5B) and both ASA and SACa (Figs. 6A and 2C). The SACa treatment (Fig. 6C) showed the spongiest appearance in the cell membrane which can be attributed to the changes in cell cytology leading to death by apoptosis. At higher magnification, some of the HT-1080 cells showed clear blebbing on the cell surface which is a key feature of cells undergoing apoptosis (Fig. 6D).

Increasing salicylates concentration to 2 mM (Fig. 7) gave similar morphological features but with higher frequencies. The ASA-treated sample at this concentration had more rounded cells and apoptotic bodies than at 0.4 mM and cells differed in their shapes from one another. For example, numerous of these cells were flattened, some were rounded and smooth and others rounded but retained their microvilli on their surface (Fig. 7A). A very distinctive change in cell structure was seen in 2 mM ASCa treated cells in which all of the cell population lost their pseudopodial attachments, anchors and curled up (Fig. 7B). The cells also had smooth membrane surface, indicating the loss of microvilli and reduced significantly in size which increased the distance between them. This was an indication of the significant level of apoptosis undergone by those cells which also reflected the role of ASCa as an antiproliferative agent at this concentration. Samples treated with 2 mM SACa also showed the presence of apoptosis as indicated by the morphology of cells (Fig. 7C).

At higher concentrations (6 and 8 mM), treated HT-1080 cells changed significantly in their morphology compared with the control (Fig. 5) and lower concentrations (Figs. 6 and 7). Salicylates treated samples with these concentrations (6 mM) large number of cells lost their anchors and microvilli and reduced significantly in size (Fig. 8A–C). Fig. 8A–C also shows the formation of apoptotic bodies. In addition to changes of HT-1080 cells towards apoptosis at 6 mM salicylates, Fig. 8D–F also shows blebbings emerging from the cell surfaces which is considered as the main feature for cells undergoing apoptotic cell death. Furthermore, HT-1080 cells also appeared round in shape, lacked of

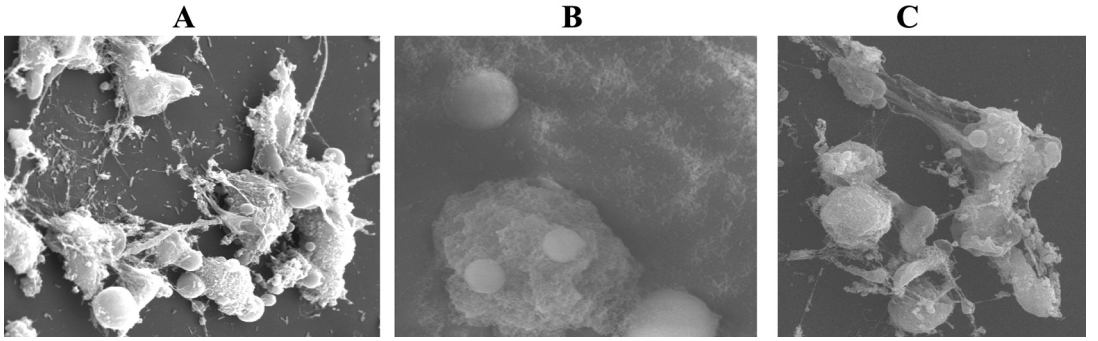


Fig. 7. The combined effect of salicylates and 200 μM BrdU on the morphology of HT-1080 cells cultured for 48 h, (A) 2 mM ASA, (B) 2 mM ASCa, (C) 2 mM SACa (magnifications A–C = 500×).

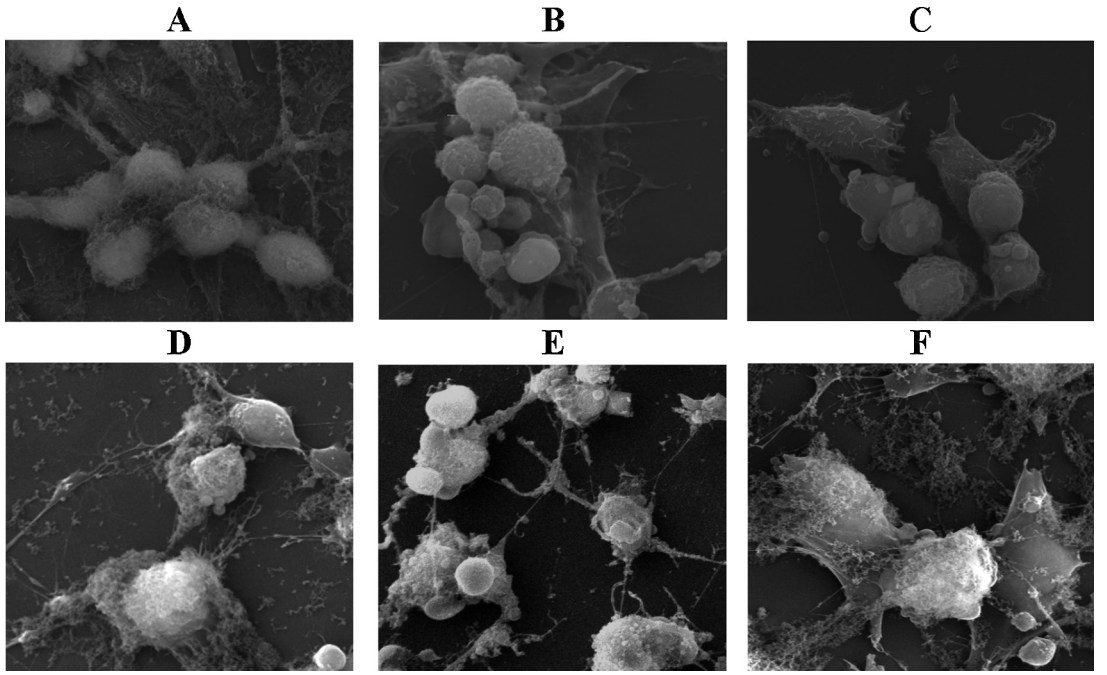


Fig. 8. The combined effect of salicylates and 200 μM BrdU on the morphology of HT-1080 cells cultured for 48 h, (A) 6 mM ASA, (B) 6 mM ASCa, (C) 6 mM SACa. (D) 8 mM ASA, (E) 8 mM ASCa, (F) 8 mM SACa (magnifications A,C–F = 500×, B = 550×).

anchors and decreased significantly in size in comparison to the control and the corresponding treatments at lower concentrations.

4. Discussion

Salicylates have been extensively studied as chemopreventative and apoptotic-inducer drugs in cancer cells [21]. The bioactivity of salicylates not only inhibit prostaglandin synthesis, but also target mitochondria and L-type Ca^{2+} signalling channels, a pathway that is upregulated in human cancer non-excitable cells. Salicylates modulate proinflammatory mediator release in mast cells *via* cyclooxygenase-independent mechanisms with the aid of Ca^{2+} signalling [22,23]. These studies indicated that ASA either enhances (at $\leq$0.3 mM) or inhibits (at >1 mM) Ca^{2+} influx [22]. In addition, our previous results have shown evidence of the pro-apoptotic activity of SACa in HT-1080 cells at 0.4 mM and modulation of both pro- and anti-apoptotic proteins. At 0.4 mM SACa up-regulated the expression of p53, p21 and Bax, and down-regulated the anti-apoptotic protein, Bcl-2 [9]. These results are likely to indicate that ASCa induces the activation of the intrinsic apoptotic pathway, which involves the activation of mitochondria. In addition, the loss of the transmembrane potential for inner membrane in mitochondria causes the release of pro-apoptotic proteins into the cytosol which subsequently activate caspases to mediate the destruction of the cell [24,25]. In addition, this current study involves assessing the cytotoxicity of ASA, ASCa and SACa at higher concentrations (0–8 mM) and in the presence of BrdU in HT-1080 human fibrosarcoma cells. BrdU is a common reagent used to measure cell proliferation, as it is readily incorporated in the *de novo* DNA synthesis during S-phase of cell cycle in place

of thymidine of newly treated cells [26,27]. Therefore, it was interesting to see the combined effect of individual salicylate and BrdU on mainly the ultrastructure of HT-1080 by SEM.

The combination of BrdU with different salicylates (ASA, ASCa and SACa) concentrations (0.4, 0.8, 2, 4, 6, 8 mM) showed dual cytotoxic effects on metabolic activities of HT-1080 cells. 0.4–2 mM salicylates showed mitogenic effects particularly when HT-1080 cells were treated for 24 and 48 h, but not at 72 h. However, at higher concentrations (4–8 mM) particularly after 48 h and 72 h, the metabolic activities of HT-1080 cells decreased in time and concentration-dependent manners. These results may indicate that salicylates exert their anti-metabolic activities after BrdU was consumed by HT-1080. Research has shown BrdU activated DNA damage signalling responses and expressed wild-type p53, induces a DNA damage response and reduced proliferation in A549 lung cancer cells [28]. However, the presence of 6 mM salicylates arrested the cell cycle at G0/G1 phase by approximately 17% compared to the control sample which is in agreement with the previous results when only 0.4 mM SACa (no BrdU) was incorporated with HT-1080 cells at 0.4 and 0.8 mM [9]. These results support the cytotoxic potential of salicylates to induce apoptosis, which was measured morphologically. The treatment of HT-1080 cells with different concentrations of ASA, ASCa and SACa in presence of BrdU showed significant ultrastructure changes, compared to the control sample. It is interesting to observe the morphological changes treated HT-1080 cells with BrdU. These treated cells curled up and exhibited a more round and swollen shape. Similar changes were reported in BrdU-treated A549 lung cancer cells after 7 days, there was an increased in the size, granularity and beta-galactosidase activity of these cells. In addition, BrdU upregulated the activation of Chk1, Chk2 and p53 in lung cells [28].

The combined effect of salicylates and BrdU on the morphology of HT-1080 cells caused cells to curl up and show apoptotic features. The percentage of apoptosis significantly increased from 6% in lower salicylate concentration to 100% as salicylate concentration increased to 8 mM. SEM of salicylate-treated HT-1080 cells at concentrations above 4 mM showed distinguishable apoptotic morphological characteristics, including cell blebbings, presence of apoptotic bodies, roundness of cells and loss of pseudopodial attachments and anchors. In addition, the cells changed shape becoming smaller and having a smoother surface when compared to the control. These morphological features give good evidence for the ability of salicylate to induce apoptosis.

5. Conclusion

Results here demonstrate that ASA, ASCa and SACa provided mainly morphological evidence of induced apoptosis in HT-1080 cells. The cytotoxicity of these salicylates and the induction of apoptotic cell death were in concentration-dependent manner, which further contributes to the development of these compounds to chemotherapy for cancer.

Conflict of interest

Authors declare that there is no any conflict of interests.

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